

# A role for the Cdc14-family phosphatase Flp1p at the end of the cell cycle in controlling the rapid degradation of the mitotic inducer Cdc25p in fission yeast

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Accepted 16 January 2004

Journal of Cell Science 117, 2461-2468 Published by The Company of Biologists 2004  
doi:10.1242/jcs.01107

## Summary

The *Schizosaccharomyces pombe* protein Flp1p belongs to a conserved family of serine-threonine-phosphatases. The founding member of this family, *Saccharomyces cerevisiae* Cdc14p, is required for inactivation of mitotic CDKs and reversal of CDK mediated phosphorylation at the end of mitosis, thereby bringing about the M-G1 transition. Initial studies of Flp1p suggest that it may play a different role to Cdc14p. Here we show that Flp1p is required for rapid degradation of the mitotic inducer Cdc25p at the end of

mitosis, and that Cdc25p is a substrate of Flp1p in vitro. Down-regulation of Cdc25p activity by Flp1p may ensure a prompt inactivation of mitotic CDK complexes to trigger cell division. Our results suggest a regulatory mechanism, and a universal role, for Cdc14p like proteins in coordination of cytokinesis with other cell cycle events.

Key words: *flp1*<sup>+</sup>, Cell cycle, Mitotic exit, Fission yeast

## Introduction

Exit from mitosis in eukaryotic cells requires the inactivation of mitotic cyclin-dependent kinases (CDKs) (Morgan, 1999; Bardin and Amon, 2001). In budding yeast, Cdc14p inactivates CDKs by promoting B-type mitotic cyclin degradation and accumulation of the CDK inhibitor Sic1p (Visintin et al., 1998; Jaspersen et al., 1999). The *CDC14* gene is essential for cell viability, and thermosensitive mutants arrest cell cycle progression in telophase with high levels of CDK1/Clb-associated kinase activity (Visintin et al., 1998; Wan et al., 1992; Taylor et al., 1997). Cdc14p is not only required for mitotic exit (Jaspersen et al., 1999; Visintin et al., 1998) but also plays a role in cytokinesis (Mensen et al., 2001; Luca et al., 2001; Yoshida et al., 2001). Cdc14p is regulated in part by changes in its subcellular localisation. It is sequestered in the nucleolus from late telophase to the metaphase-anaphase transition of the following cell cycle by the Net1p/Cfi1p protein of the RENT complex (Regulator of Nucleolar Silencing and Telophase) (Visintin et al., 1999; Straight et al., 1999; Traverso et al., 2001), from where it is released during anaphase, dependent upon the FEAR (Cdc Fourteen Early Anaphase Release) and MEN (Mitotic Exit Network) group of proteins (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999; Stegmeier et al., 2002). Budding yeast Cdc14 is regarded as the effector of the MEN signal network that inactivates mitotic cyclin-dependent kinases (mitotic CDKs) at the end of anaphase so that cells can exit mitosis (Traverso et al., 2001).

Cdc14 orthologues have been identified in other species including *Schizosaccharomyces pombe*, *Caenorhabditis*

*elegans* and *Homo sapiens* on the basis of sequence similarity to the budding yeast *Saccharomyces cerevisiae* protein (Li et al., 1997; Cueille et al., 2001; Trautmann et al., 2001; Gruneberg et al., 2002). (All) Cdc14 proteins characterised to date are dual-specificity protein phosphatases that share a core of approximately 300 amino acids towards the amino terminus that includes a strictly conserved protein-phosphatase domain motif (Gray et al., 2003).

In fission yeast, the Cdc14-related protein phosphatase Flp1p (also named Clp1) seems to perform a different role in cell cycle progression to that of its *S. cerevisiae* counterpart. A null mutant of the *S. pombe flp1* is viable but divides at a reduced size and has defects in septation (Cueille et al., 2001; Trautmann et al., 2001). In contrast to Cdc14p, Flp1p is not essential for mitotic exit. Consistent with this, Flp1p is not required for the dephosphorylation and activation of APC activator Ste9p, nor for the accumulation of the cyclin-dependent kinase inhibitor Rum1p, nor for the destruction of the mitotic B-type cyclin Cdc13p (Cueille et al., 2001). Although both Flp1p and Cdc14p proteins are nucleolar-located in interphase, from where they are released to the nucleus at mitosis, Flp1p it is also present on the spindle pole body during interphase as well as on the spindle and the contractile ring during mitosis, although its substrates at these locations are unknown (Cueille et al., 2001; Trautmann et al., 2001). The regulation of Flp1p subcellular localisation is complex and differs in details from the mechanisms regulating Cdc14p localisation in *S. cerevisiae*. Budding yeast Cdc14p nucleolar release requires MEN function. In fission yeast the

orthologues of the MEN genes are collectively termed the SIN (Septation Initiation Network) (Le Goff et al., 1999; Balasubramanian et al., 2000). Recently, we have shown that SIN function is not required to release Flp1p from the nucleolus in *S. pombe*, although inactivation or attenuation of the SIN is needed for the relocalisation of Flp1p proper to the nucleolus at the end of mitosis (Cueille et al., 2001).

The fact that *flp1Δ* mutant cells are advanced in mitosis suggests an interaction with the proteins controlling mitosis. This is supported by the finding that overexpression of *flp1+* arrests cells in G2 with dephosphorylated Cdc25p, and that this arrest depends upon active *wee1* (Cueille et al., 2001). These studies suggested that elements of the mitotic control system (Cdc25p and Wee1p) or their upstream regulators might be of the targets of Flp1p (Cueille et al., 2001; Trautmann et al., 2001). We have explored this further, and examined the role of Flp1p in regulating the phosphoprotein phosphatase Cdc25p.

## Materials and Methods

### Strains and cell synchronisation

Standard molecular biology and genetic methods were used as described previously (Moreno et al., 1991; Norbury and Moreno, 1997). Yeast transformation was carried out using the lithium acetate transformation protocol (Norbury and Moreno, 1997). Fission yeast were grown and manipulated according to standard protocols in either yeast extract (YE) or minimal (M) medium, containing appropriate supplements [YES media: yeast extract plus supplements (225 mg/l adenine, histidine, leucine, uracil and lysine hydrochloride) (Moreno et al., 1991)]. *S. pombe* strains used in this study were from Moreno, Simanis and Bueno's laboratory collections, in particular, all strains involving the *flp1Δ* deletion carried the construction described previously (Cueille et al., 2001). The induction of synchrony by *nda3-KM311* was performed by blocking cells at metaphase by incubating them for 6 hours at 19°C or after elutriation of G2 cells by incubating them for 4 hours at 20°C (cold sensitive arrest or *cs* arrest). Under these conditions more than 80% of cells blocked at metaphase. Alternatively, to obtain a better degree of synchrony and prior to the *cs* arrest, *nda3-KM311* cells were blocked for 3 hours with hydroxyurea (HU). For induction of the *nmt1* promoter, a culture growing exponentially in medium containing thiamine was washed twice and resuspended in medium without thiamine as described previously (Cueille et al., 2001).

### Oligonucleotides used for cloning of the *GST-flp1+* and *GST-flp1CS* genes and construction of *nmt:GST-flp1*-containing plasmids

To construct the GST derivatives used in this work different PCRs were performed to amplify the full ORF of *flp1+* and for creation of the *flp1CS* (C286S) mutant with standard cloning and site-direct mutagenesis methods. After cloning, all plasmids generated were sequenced to check that not other changes than the desired one was introduced in the ORF by PCR. Oligonucleotides used for cloning of *GST-flp1+* or *GST-flp1CS* were:

5'-TACTTGTCATATGGATTACCAAGA-3' where the underlined sequence is an *NdeI* site for cloning.

5'-AAAACTGCGGCCGCCATTTAAACCAG-3' where the underlined sequence is a *NotI* site for cloning.

Oligonucleotides used for mutagenesis (to obtain *flp1CS*) were: 5'-GCTGTTTCATAGCAAAGCAGG-3' and 5'-CCTGCTTGC-TATGAACAGC-3'.

*nmt:GST-flp1*-containing plasmids were constructed as described previously (Shiozaki and Russell, 1997).

### Antisera and tagged strains

Antisera and tagged strains allowing detection of Cdc25p, Cdc13p, Cdc2p, Wee1p and tubulin have been described previously (Bähler and Pringle, 1998; Blanco et al., 2000; Cueille et al., 2001).

### Cell extracts, immunoprecipitation, phosphatase assays, kinase analysis and western blot analysis

Cell extracts for western blotting and immunoprecipitation were obtained as described previously (Cueille et al., 2001). For western blots, 75 mg of total protein extract was run on a 12% standard SDS-PAGE gel, transferred to nitrocellulose and probed with rabbit affinity purified anti-Cdc25 (1:250), SP4 anti-cdc13 (1:250) and anti-Cdc2 (1:500) polyclonal antibodies or anti-haemagglutinin (HA) (12CA5, Roche) and anti-myc monoclonal antibodies. Secondary antibodies used were goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase (Amersham) (1:3500). Mouse TAT1 anti-tubulin monoclonal antibodies (1:500) and goat anti-mouse conjugated to horseradish peroxidase (1:2000) as secondary antibody was used to detect tubulin as a loading control. Immunoblots were developed using the ECL kit (Amersham) or Super Signal (Pierce). GST purification has been described previously (Shiozaki and Russell, 1997). Phosphatase assays were performed with para-nitrophenylphosphate (pNPP), immunoprecipitated Cdc25p or GST-Cdc25p phosphorylated in vitro as substrates by incubation in phosphatase buffer (imidazole-HCl 50 mM, pH 6.6, EDTA 1 mM, DTT 1 mM) with purified GST-Flp1p (and appropriate controls) for 40 minutes at 30°C (or as indicated). Kinase assays were performed with H1 histone (Calbiochem) or bacterially produced GST-Cdc25p as substrates on immunoprecipitates at 32°C as described previously (Moreno et al., 1989; Calzada et al., 2000), and quantified with a phosphorimager. H1 histone signals were normalised to the *nda3-KM311* arrest level.

### Microscopy, cytology and flow cytometry

Approximately 10<sup>7</sup> cells were collected by centrifugation, washed once with water, fixed in 70% ethanol and processed for flow cytometry or DAPI staining, as described previously (Moreno et al., 1991).

The mitotic index was determined from the percentage of binucleate cells (cells with two nuclei and without a septum) after DAPI staining. The septation index was determined from the percentage of cells with a septum after calcofluor staining. Also, to evaluate the completion of the cell cycle, the percentage of cells in anaphase B was estimated by counting cells with two nuclei and an elongated spindle. Staining of microtubules was performed as described previously (Bähler and Pringle, 1998; Balasubramanian et al., 1997) using anti-tubulin antibody TAT-1 (Bähler and Pringle, 1998) or using a *GFP-atb2+* strain (Yamamoto et al., 1999). Staining of myc<sub>12</sub>-Cdc25p protein was performed as described previously (López-Girona et al., 1999). Fluorescence images were collected using a Zeiss Axioplan 2 microscope with a 63× or a 100× objective and a digital camera (Hamamatsu ORCA-ER) and processed with Improvision software. More than 1000 cells were examined for each time point, and each experiment was repeated at least three times, to gain an estimate of error.

## Results

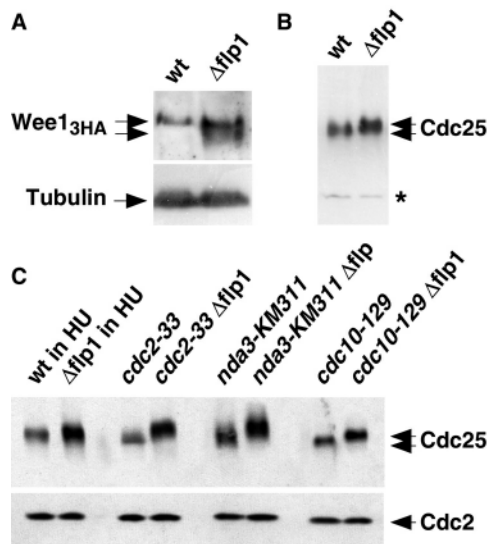
### Cdc25p accumulates in a *flp1* null *S. pombe* mutant

In *S. pombe* cells, as well as in most eukaryotes, entry into mitosis is controlled by the balance between the activities of the inhibitory kinase Wee1p and the opposing effect of the phosphatase Cdc25p in regulating the activity of Cdc2p (Russell and Nurse, 1986; Russell and Nurse, 1987a; Gould

and Nurse, 1989; Norbury et al., 1991; Norbury and Nurse, 1992; McGowan et al., 1993), the catalytic subunit of CDK-complexes. We therefore examined the consequences of mutating the *flp1* gene upon the stability and phosphorylation state of Cdc25p and Wee1p. Deletion of the *flp1* gene resulted in the accumulation of hyperphosphorylated Cdc25p (Fig. 1). This pattern was independent of the stage of the cell cycle, given that cells blocked in G1, S, G2 phase or mitosis accumulated hyperphosphorylated Cdc25p when *flp1* was mutated (Fig. 1), which is consistent with the idea that Flp1 may influence the phosphorylation state of Cdc25p. However, Wee1p was partially dephosphorylated in *flp1* mutant strains (Fig. 1), indicating that Flp1p does not play an essential role in Wee1p dephosphorylation.

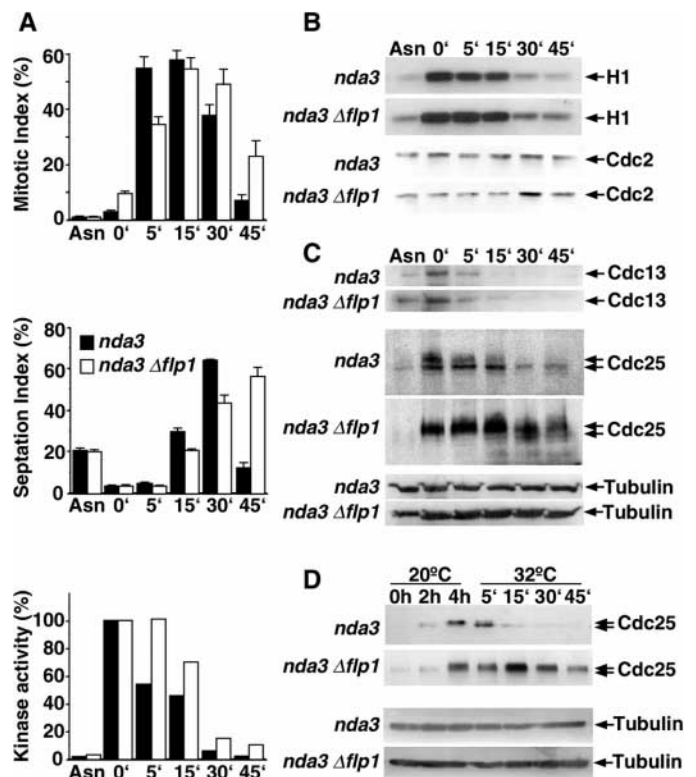
#### Cdc25p proteolysis is delayed in *flp1*Δ mutant fission yeast cells at the M to G1 transition

Though Flp1p is not essential for completion of mitosis (Cueille et al., 2001; Trautmann et al., 2001), we examined whether *flp1* null cells showed any defects in mitotic exit. We therefore analysed the M to G1 transition in cells synchronised by *nda3-KM311* arrest release. Exponentially growing cultures of *nda3-KM311* and isogenic *nda3-KM311 flp1*Δ were blocked at 19°C for 6 hours and released by transfer to 32°C. Samples



**Fig. 1.** Wee1p and Cdc25p proteins in *flp1*Δ mutant *S. pombe* cells. (A) Wee1p is partially dephosphorylated in a *flp1* mutant strain. Cells from asynchronous cultures of *wee13HA* and *wee13HA flp1*Δ (incubated at 30°C) were processed for western analysis and probed with α-Ha monoclonal or α-tubulin antibodies. (B) Cdc25p is hyperphosphorylated in the *flp1* deletion strain. Cells from asynchronous cultures of *wt* and *flp1*Δ (incubated at 30°C) were processed for western analysis and probed with α-Cdc25 polyclonal antibodies. The asterisk marks a reference protein that cross-reacts with the antibody. (C) Cdc25p is hyperphosphorylated in *flp1* deletion strains at different stages of the cell cycle. Western blot analysis of Cdc25p protein extracts from cells block in S phase (hydroxyurea, HU, 4 hours), in G2 (*cdc2-33*, 4 hours at 35.5°C), in mitosis (*nda3-KM311*, 6 hours at 19°C) or in G1 (*cdc10-129*, 4 hours at 35.5°C). Blots were probed with α-Cdc25 and α-Cdc2 polyclonal antibodies, the latter for a loading and proper migration control.

were taken at intervals and processed for cytological observation, western blot analysis and assayed for Cdc13-associated kinase activity (Fig. 2). In *nda3-KM311 flp1*Δ mutants the rate of disappearance of binucleate cells (mitotic index) (Fig. 2A, upper plot) was delayed compared with the control. Consistent with this, the appearance of septated cells was also delayed in *flp1* mutant cells (Fig. 2A, middle plot). However, analysis of the exit from mitosis by measuring the rate of disappearance of binucleated cells with elongated spindles (anaphase B cells) showed no major differences between *flp1* mutants and controls (data not shown). Therefore, we conclude that the delay of disappearance of binucleate cells in strains deleted for *flp1* is the result of the delay in cytokinesis. Though the levels of the Cdc13-cyclin B protein declined similarly in the control and the *flp1* deletion mutant as cells exit from mitosis, following release from the



**Fig. 2.** Cell division is delayed in *flp1*Δ mutant *S. pombe* cells. *nda3-KM311* and *nda3-KM311 flp1*Δ (otherwise isogenic strains) were blocked in metaphase by incubation of asynchronously growing cultures (Asn) at 20°C for 6 hours. Cells were then released from the block at 32°C and samples were taken at the indicated intervals and processed for cytological and biochemical analysis. (A) Plots of binucleate (top plot, Mitotic Index), septated cells (middle plot, Septation Index) and relative kinase activity (lower plot) are shown. (B) Cdc13p-associated H1 kinase activity (top panels) and relative amounts of immunoprecipitated Cdc2 for the kinase assays (lower panels). (C) Cdc13p, Cdc25p and α-tubulin western analysis (as indicated) from samples processed from *nda3-KM311* and *nda3-KM311 flp1*Δ cells. (D) Elutriated G2 *nda3-KM311* (control) or *nda3-KM311 flp1*Δ cells were blocked at metaphase by incubating them for 4 hours at 20°C and then released at 32°C. Samples were taken at the indicated intervals and processed for α-tubulin and Cdc25p western analysis.

metaphase arrest, the activity of Cdc13-associated H1 kinase declined faster in the *nda3-KM311* control than in *nda3-KM311 flp1Δ* double mutant (Fig. 2A, lower plot, and 2B). Thus, Flp1p may contribute to the prompt inactivation of CDK as cells initiate anaphase.

In wild-type cells, Cdc25p is rapidly dephosphorylated, and then degraded during mitotic exit (Fig. 2C). The protein then reaccumulates during S and G2 phases, reaching a peak as cells enter mitosis (Moreno et al., 1990; Ducommun et al., 1990). In contrast, upon release of a *nda3-KM311 flp1Δ* mutant, Cdc25p remained phosphorylated and its steady state level did not change significantly up to 30 minutes after the release from the metaphase block (Fig. 2C).

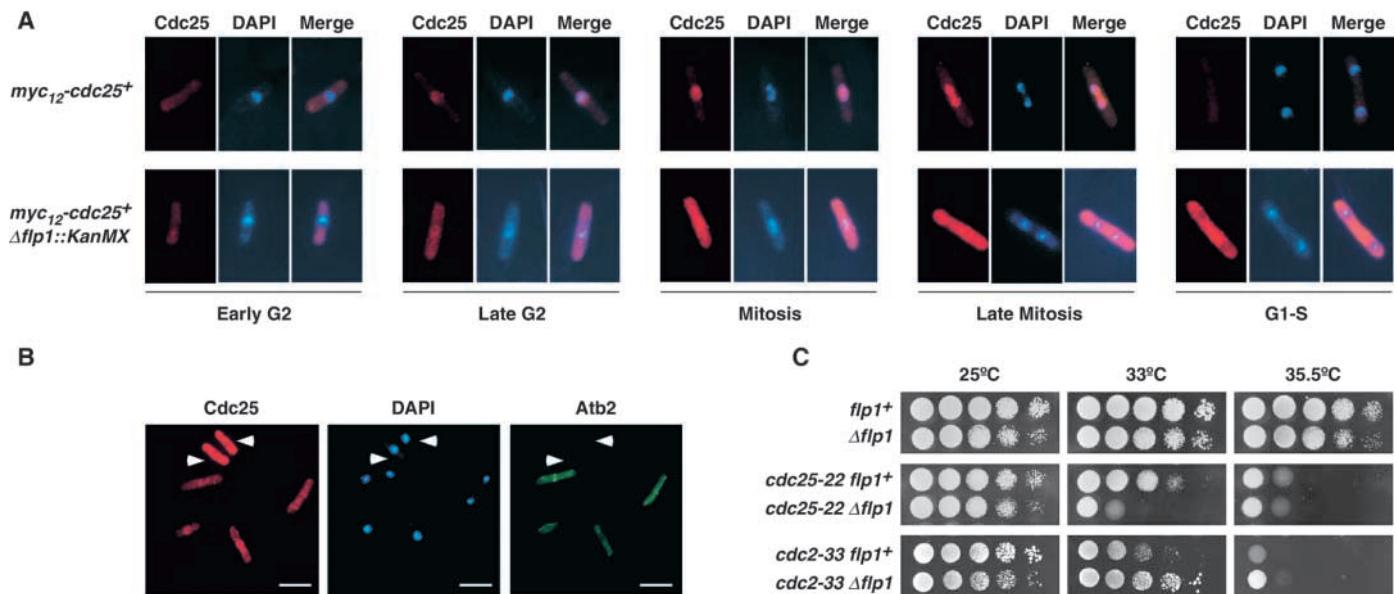
To obtain better synchrony, this result was confirmed using a culture of *nda3-KM311* generated by centrifugal elutriation, which allowed us to obtain over 90% of cells in metaphase after 4 hours at 20°C. This experiment confirmed that p80<sup>*cdc25*</sup> remains phosphorylated longer and is more stable in the absence of *flp1* function (Fig. 2D). Together, these results strongly suggest that rapid degradation of the CDK activator Cdc25p at the end of mitosis is regulated by the Flp1p phosphoprotein phosphatase.

#### Cdc25p localisation is altered in *flp1Δ* mutants

Next, we analysed the localisation of the Cdc25p protein in *flp1Δ* deletion mutant cells expressing Myc-tagged Cdc25p by indirect immunofluorescence microscopy. As described previously, in the *myc12-cdc25<sup>+</sup>* control the amount of nuclear

Cdc25p increased as cells progress throughout G2, to be mostly nuclear in late G2 and mitosis and then decreased in the transition of mitosis to G1 (López-Girona et al., 1999) (Fig. 3A, upper panels). We observed that in *myc12-cdc25<sup>+</sup> flp1Δ* mutant cells the signal of Cdc25p was clearly more intense throughout the cell, in both cytoplasm and nuclei, in every cell cycle stage, indicating that deletion of *flp1* causes a higher stability and inappropriate localisation of the Tyr15 phosphatase from late G2 to the G1/S transition in fission yeast cells (Fig. 3A, lower panels). To ensure that *flp1Δ* mutants accumulated more Cdc25p protein than wild-type, both types of cells were mixed and co-prepared for immunofluorescence (Fig. 3B). In these experiments the *myc12-cdc25<sup>+</sup>* control was also labelled with *GFP-atb2<sup>+</sup>* (Yamamoto et al., 1999) to distinguish them from *myc12-cdc25<sup>+</sup> flp1Δ* mutant cells. This approach confirmed that Cdc25p is more stable in *S. pombe* cells lacking the Cdc14p-related phosphatase.

Consistent with a higher stability of active (hyperphosphorylated) Cdc25p in the absence of Flp1p, deletion of the *flp1* gene partially rescued the *ts* defect of *cdc2-33* cells, a *cdc2<sup>ts</sup>* allele that causes a tight G2 arrest with low CDK-associated kinase activity (Carr et al., 1989; Castellanos et al., 1996), allowing them to form colonies up to 33°C (Fig. 3C). Conversely, we found that *flp1Δ* is synthetically lethal at semi-permissive temperatures when combined with *cdc25-22 ts* mutants. A *flp1Δ cdc25-22* strain is inviable at 33°C (Fig. 3C), a temperature at which both *flp1Δ* and *cdc25-22* single mutants grew to form colonies. *cdc25-22 flp1Δ* double mutants arrested at 33°C with a similar *cdc* arrest phenotype to that



**Fig. 3.** Cdc25p localisation and stability are altered in *flp1Δ* mutant fission yeast cells. (A) Asynchronous exponentially growing cultures of *myc12-cdc25* (reference control) and *flp1Δ myc12-cdc25*, otherwise isogenic strains, were processed for indirect immunofluorescence microscopy using anti-Myc antibodies. Nuclei were stained with DAPI. Approximate phase of the cell division cycle is indicated. Cells at different stages of the cell cycle were scored to quantify the defect observed in proper Cdc25p localisation as compared with the reference control (*myc12-cdc25* cells). (B) Equal numbers of cells from asynchronous cultures of two different strains (*myc12-cdc25 GFP-atb2<sup>+</sup>* and *flp1Δ myc12-cdc25*) were mixed and processed for indirect immunofluorescence microscopy using anti-Myc antibodies, DAPI and GFP-Atb2 (the latter to distinguish *flp1<sup>+</sup>* wild-type from *flp1Δ* mutant). *flp1Δ* mutant cells are indicated by white arrowheads in the photomicrographs. Scale bars: 10 μm. Note that GFP-Atb2p-positive cells contain less Myc-Cdc25p (as judged by the relative intensity of anti-Myc staining). (C) Ten-fold dilution of the indicated strains from log-phase cultures at 25°C were inoculated in YES Petri dishes and incubated for 48 hours at the indicated temperatures.

of *cdc25-22* at 35.5°C (Russell and Nurse, 1986). These observations are consistent with data indicating that cells lacking Flp1 are defective in regulating the levels and localisation of Cdc25p (Fig. 2 and Fig. 3A), and favour the hypothesis that Flp1p controls the steady state levels of Cdc25p.

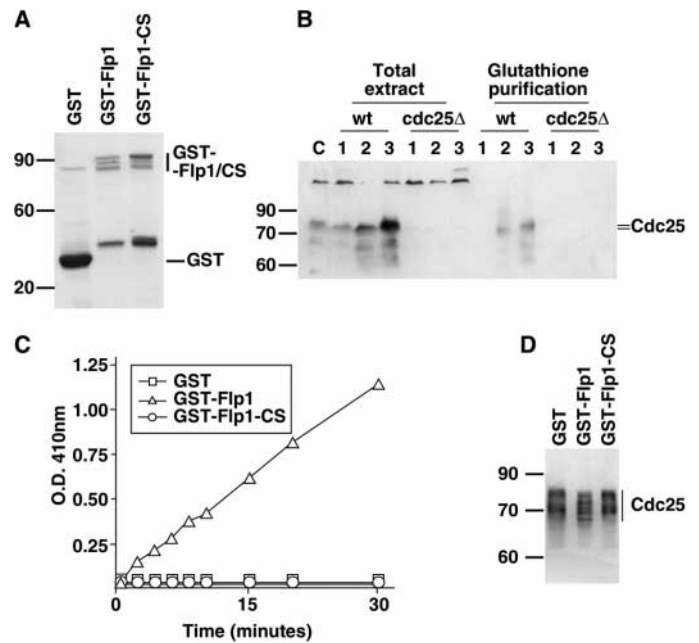
The Flp1 protein is a phosphatase that interacts in vivo with Cdc25p

Since Cdc25p was not promptly dephosphorylated and degraded during mitotic exit in the *flp1::kanMX6* mutant, we next performed a biochemical analysis to determine whether p60<sup>Flp1</sup> could dephosphorylate p80<sup>cdc25</sup> in vitro. We constructed a vector to produce full-length Flp1p (or a mutant form in which the cysteine 286 was changed to serine: Flp1pC286S) as a fusion protein with glutathione S-transferase under the control of the thiamine repressible *nmt1* promoter. We expressed this *nmt1:GST-flp1*<sup>+</sup> fusion and found that it induced a G2 cell cycle arrest similar to *nmt1:flp1*<sup>+</sup>-expressing cells, indicating that GST-Flp1p is functional in vivo. In contrast to our previous report (Cueille et al., 2001), we found that expression of *nmt1:GST-flp1CS* also caused cells to arrest in G2, but the block was leaky and delayed by approximately 4 hours as compared to a *nmt1:flp1*<sup>+</sup> control. The difference may reflect the time of expression used.

GSTp (control), GST-Flp1p or GST-Flp1CSp were purified virtually to homogeneity from *S. pombe* cells after 20 hours of induction (Fig. 4A). Three high molecular mass GST-Flp1p species were consistently observed. How these differ is presently unclear, but they are likely to be either differentially phosphorylated forms of the fusion protein or degradation products.

To address whether Cdc25p and Flp1p proteins interact in vivo we tested for the presence of Cdc25p in GST-Flp1p protein samples after the purification with glutathione-Sepharose (GSH) beads. By western blot analysis, we found that GST-Flp1p and GST-Flp1CSp, but not GSTp, associate in vivo with Cdc25p (Fig. 4B). Significantly, the Cdc25p protein that associates with GST-Flp1CSp mutant form lacking phosphatase activity is hyperphosphorylated.

With the purified proteins from *cdc25Δ cdc2-3w* (*cdc25* deleted strains) we next tested the ability of GST-fused proteins to function in an in vitro phosphatase assay using a standard substrate. We found that GST-Flp1p dephosphorylated para-nitrophenylphosphate (pNPP) (Fig. 4C). The dephosphorylation reaction was linear during the time course. Under similar conditions of protein or substrate concentration and time, we did not detect any phosphatase activity either with the GST protein alone or with GST-Flp1CSp mutant control, indicating that Flp1p has intrinsic phosphatase activity. To perform a phosphatase assay with Cdc25p as substrate we isolated phosphorylated p80<sup>cdc25</sup> from mitotic cells. A cold-sensitive *nda3* strain was arrested in metaphase at the restrictive temperature, cell extracts were prepared and protein samples immunoprecipitated with a polyclonal antibody raised against Cdc25p. Washed immunoprecipitates were incubated in the presence of either GSTp, GST-Flp1p or GST-Flp1CSp and subjected to western analysis to detect Cdc25p after denaturing SDS gel electrophoresis. As shown (Fig. 4D), GST-Flp1p, but not GST-Flp1CSp, partially dephosphorylates



**Fig. 4.** Flp1p interacts with and dephosphorylates Cdc25p.

(A) GSTp, GST-Flp1p and GST-Flp1CSp proteins were expressed under the control of the *nmt1* promoter in a *cdc25Δ cdc2-3w* strain and purified with glutathione-Sepharose (GSH) beads. An aliquot of each sample was processed for PAGE and stained with Coomassie Blue to visualise proteins. (B) GST-Flp1p and GST-Flp1CSp but not GSTp associate in vivo with Cdc25p. GSTp (negative control, lanes 1), GST-Flp1p (lanes 2) or GST-Flp1CSp (lanes 3) were purified and processed for the detection of Cdc25p from *cdc25*<sup>+</sup> (wt) or *cdc25Δ cdc2-3w* (*cdc25Δ*) cells expressing them (20 hours of induction of the *nmt1* promoter). A total extract control from *nda3-KM311* metaphase-arrested cells is shown for the detection of Cdc25p (lane C). Note that the Cdc25p protein that associates with the GST-Flp1CSp form is hyperphosphorylated as compared with the migration of Cdc25 from metaphase-arrested cells shown in lane C. (C,D) Purified GSTp, GST-Flp1p and GST-Flp1CSp proteins from A were assayed in vitro for their ability to dephosphorylate an artificial substrate, pNPP (C), or Cdc25p immunoprecipitated from *nda3-KM311* metaphase-arrested cells (D). Note in D the mobility shift observed when immunoprecipitated Cdc25p is incubated with GST-Flp1p, in comparison with GSTp and GST-Flp1CSp negative controls.

Cdc25p protein. The incomplete dephosphorylation of Cdc25p could be due to either low efficiency of GST-Flp1p or, more likely, to dephosphorylation of specific phosphorylated residues in Cdc25p, since the phosphorylation pattern did not change with time.

Using a different approach to assess whether Cdc25p is a substrate of the Flp1p phosphatase, we developed an assay in which purified GST-Cdc25p from bacteria was first phosphorylated by immunoprecipitated Cdc2/Cdc13 (CDK) complexes and then used as a substrate for purified GST-Flp1p from fission yeast with appropriate controls (Fig. 5). In this assay even the lowest quantities of GST-Flp1p used were able to partially dephosphorylate CDK-phosphorylated GST-Cdc25p (plot in Fig. 5). These data are consistent with those shown in Fig. 4, and together, they support the hypothesis that Flp1p may regulate Cdc25p in vivo directly by dephosphorylation.

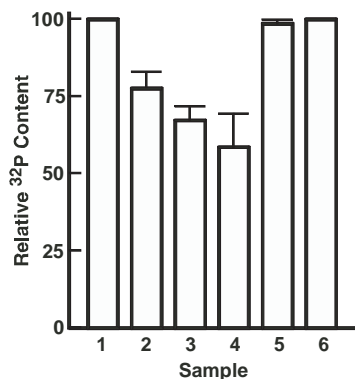
### Loss of *flp1* does not alter Wee1p phosphorylation as *S. pombe* cells exit from mitosis

Having shown that Flp1p controls the level of the CDK-activator Cdc25p, we then performed a metaphase-block and -release analysis with *nda3-KM311 wee1<sub>3HA</sub>* and isogenic *nda3-KM311 flp1Δ wee1<sub>3HA</sub>* in order to test whether the Wee1p protein kinase was also regulated by the Flp1p phosphatase at the end of mitosis (Fig. 6A). We did not detect any changes either in the steady state level of Wee1p, or in its gel mobility compared to the *nda3-KM311 wee1<sub>3HA</sub>* control, suggesting that Flp1p does not dephosphorylate Wee1p at the M-G1 transition.

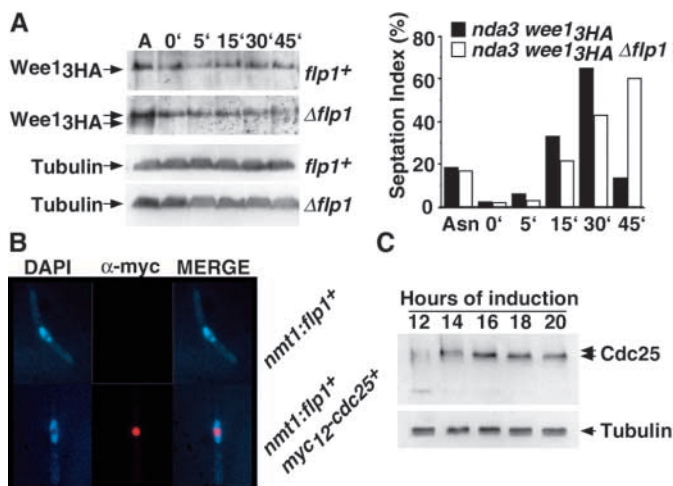
Finally, to examine further why over-expression of *flp1<sup>+</sup>* causes cells to arrest in G2 with the accumulation of a hypophosphorylated, and most likely, inactive form of Cdc25p (Fig. 6), we analysed the localisation of the Cdc25p protein in these cells. Interestingly, most of the Cdc25p was sequestered in the nucleolus (Fig. 6B), perhaps providing an explanation for the cell cycle arrest. Thus, we believe that the G2 arrest may not reflect the true physiological point of action of Flp1p in the cell cycle, though confirmation of this awaits generation of a conditional allele of *flp1*.

## Discussion

An important feature of the Cdc25p mitotic inducer is its oscillatory pattern of appearance at each fission yeast cell division cycle. The *cdc25<sup>+</sup>*-encoded tyrosine phosphatase slowly accumulates during interphase to reach a critical level



**Fig. 5.** GST-Flp1p dephosphorylates in vitro CDK-phosphorylated GST-Cdc25p. Purified GST-Flp1p and GST-Flp1CSp proteins from *cdc25Δ cdc2-3w* strains (as in Fig. 4A) were assayed in vitro, in the linear range of the assay, for their ability to dephosphorylate purified GST-Cdc25p from bacteria and previously phosphorylated in vitro by immunoprecipitated Cdc2/Cdc13 complexes. The relative amount of radioactive GST-Cdc25p was quantified in three different and independent experiments (in which every component was de novo purified or immunoprecipitated) and plotted. 110 μM CDK-phosphorylated GST-Cdc25p were incubated with 0 μM (sample 1 and 6), 55 μM (sample 2), 110 μM (sample 3) or 220 μM (sample 4) of GST-Flp1p or 330 μM GST-Flp1CSp (sample 5). Samples were run on PAGE gels and stained with Coomassie Blue. Relative phosphorylation levels in GST-Cdc25p were quantitated using a phosphorimager and plotted as a percentage of the phosphorylation levels measured/observed in reactions without GST-Flp1p. Note that the extent of GST-Cdc25p dephosphorylation does increase slightly with addition of more GST-Flp1p. Bars indicate standard error.



**Fig. 6.** The phosphorylation state of Wee1p is not affected by loss of *flp1p* function. (A) A metaphase block-and-release experiment of *nda3-KM311 wee1<sub>3HA</sub>* (control) and *nda3-KM311 wee1<sub>3HA</sub> flp1Δ* (otherwise) isogenic strains. Cells were synchronously released from an *nda3* block (6 hours at 20°C), samples were taken at the indicated (in minutes) intervals to analyse Wee1p protein phosphorylation by shift mobility after western analysis with α-Ha monoclonal antibody. The plot of septated cells (Septation Index) is shown. (B) Cdc25p localises at the nucleolus when *flp1<sup>+</sup>* is overexpressed. Photomicrographs of *nmt1: flp1<sup>+</sup>* or *nmt1: flp1<sup>+</sup> myc12-cdc25<sup>+</sup>* cells induced for the expression of Flp1 for 18 hours. (C) Overexpression of *flp1<sup>+</sup>* induces Cdc25p dephosphorylation. Expression of Flp1 was induced in an *nmt1: flp1<sup>+</sup>* strain by removal of thiamine from the medium, samples were taken at indicated intervals and processed for western analysis and immunofluorescence (see B).

at the end of G2 (Moreno et al., 1990; Ducommun et al., 1990), to be first activated by polo, and then by Cdc2/Cdc13 kinases, to allow full activation of the latter kinase that induces entry into mitosis (Hoffmann et al., 1993; Kovelman and Russell, 1996; Glover et al., 1998; Nigg, 1998). During mitosis, Cdc25p is abruptly degraded (in a cell-cycle-dependent manner) (Moreno et al., 1990; Ducommun et al., 1990; Nefsky et al., 1996). Because of this pattern of synthesis and degradation it was suggested that mitosis is regulated by the cyclic accumulation (and activation) of the Cdc25p protein (Moreno et al., 1990). Fission yeast Cdc25p is regarded as the main effector that activates mitotic cyclin-dependent kinases at the G2/M transition.

Several lines of evidence shown in this work strongly suggest that the *flp1<sup>+</sup>*-encoded phosphatase induces the rapid degradation of the Cdc25p mitotic inducer at the end of mitosis. In this study we provide evidence that Flp1p can act as a phosphatase in vitro (Fig. 4A), and that it interacts in vivo with, and can dephosphorylate Cdc25p in vitro (Fig. 4B,D, Fig. 5). Since Cdc25p is not dephosphorylated during mitotic exit in an *flp1* null mutant (see Figs 2, 3) we propose that dephosphorylation of specific residues in Cdc25p by Flp1p may be part of the mechanism that triggers its destruction. However, it is also possible that Flp1p activates another phosphatase, which is in turn responsible for the direct dephosphorylation of Cdc25p. Future experiments to determine which CDK-phosphorylated residues of Cdc25p are substrates of Flp1p will resolve this. Alternatively, Flp1p might

regulate the activity of the degradation machinery of Cdc25p (see below).

We have suggested earlier that Flp1p does not play any role in the inactivation of mitotic cyclin-dependent kinases through Cdc13p-cyclin degradation or Rum1p-CDK inhibitor stabilisation at the end of mitosis in fission yeast (Cueille et al., 2001). In the present work we have extended the analysis of *flp1* null mutants as they exit from mitosis and observed that a major defect in *flp1* null mutant cells is the timing of Cdc25p proteolysis, which correlates with a delay in Cdc2/Cdc13 inactivation and septation (see Figs 2, 3). Although, it was known the Cdc25p is unstable (Moreno et al., 1990; Ducommun et al., 1990; Nefsky and Beach, 1996), our work suggests that proteolysis of the mitotic inducer contributes to CDK inactivation as cells exit mitosis for the proper coordination of nuclear division with cytokinesis. Additionally, the degradation of Cdc25 at the M to G1 transition may be part of the mechanism that ensures a minimum cell mass before entering mitosis in the next cell cycle (Daga and Jimenez, 1999), and since it would also [in a redundant manner with Wee1p and Rum1p (Russell and Nurse, 1987a; Moreno and Nurse, 1994)] prevent activation of any Cdc2/Cdc13 complexes that form during G1, we propose that it may also be important for proper coordination between the end of mitosis and cytokinesis in fission yeast, as it has been suggested that cytokinesis occurs only when mitotic B-type cyclin CDK activity is reduced to low levels (reviewed by Irniger, 2002; Simanis, 2003). This model does not exclude the existence of others substrates for the Flp1p phosphatase, but our results strongly suggest that an important one is the CDK activator Cdc25p.

We and others suggested earlier that Flp1p may regulate the activation of Cdc2p at the onset of mitosis by controlling Cdc25p and Wee1p (Cueille et al., 2001; Trautmann et al., 2001). This hypothesis was founded upon two observations: first the *flp1* null mutant is advanced into mitosis, and second, the G2 arrest imposed by over-expression of *flp1*<sup>+</sup>. The experiments that we present here show that in the absence of *flp1*, *S. pombe* cells initiate the cell cycle with more Cdc25p protein (Figs 2, 3). This raises the possibility that they are *wee* because they reach the threshold level of Cdc25p required for Cdc2/Cdc13 activation earlier, potentially explaining their advanced-into-mitosis (*wee*) phenotype.

Our analysis shows that Flp1p phosphatase does not play an essential role in Wee1p dephosphorylation (see Figs 1, 6). Nevertheless, we propose that one of the regulatory mechanisms that trigger the inactivation of mitotic CDKs before *S. pombe* cells undergo septation, is the shift in the balance between dephosphorylation and phosphorylation of the tyrosine 15 of Cdc2. Although our study only proves that Flp1p down regulates Cdc25p, a tyrosine kinase has to be active at this transition of the cell cycle. As fission yeast cells enter mitosis, Nim1 phosphorylates Wee1p (Russell and Nurse, 1987b) at the C-terminus to inactivate it (Mosser and Russell, 2000). Wee1p remains phosphorylated, and hence probably inactive, as *S. pombe* cells exit from mitosis (Fig. 6A). It is tempting to speculate that the Cdc2-tyrosine 15 kinase Mik1p (Lundgren et al., 1991) may play a role in inactivation of cdc2 during G1, in addition to its role during S phase (Christensen et al., 2000; Baber-Furnari et al., 2000). Whether Flp1p regulates Wee1p localisation

and/or the activation of Mik1 will be addressed in future studies.

The Cdc25p protein is ubiquitinated for proteolysis (Nefsky and Beach, 1996). Although the E3 ligase Pub1p is required for the predominant mechanism of Cdc25p polyubiquitination to be active, there is evidence for the existence of an alternative Cdc25p protein ubiquitination pathway (Nefsky and Beach, 1996). Dephosphorylation of Cdc25p may make it a target for Pub1p, or Flp1p may also control the activity of Pub1p. Alternatively, Flp1p may activate a Pub1p-independent degradation pathway of Cdc25p. Distinguishing between these alternatives will be the subject of future studies.

In many cases CDK-mediated phosphorylation of proteins implicated in cell cycle control targets them for proteolysis after polyubiquitinylation (Dshaies, 1995; Dshaies et al., 1995). In the case of the Cdc25p mitotic inducer, CDK-mediated phosphorylation is the signal to bring it to full activity (Hoffmann et al., 1993; Kovelman and Russell, 1996). Given that our *in vivo* biochemical analysis strongly suggests that once Flp1p (a Ser/Thr phosphatase) dephosphorylates Cdc25p (a Tyr phosphatase) the mitotic inducer becomes unstable, we speculate that the signal that targets Cdc25p for destruction will be given by Flp1p-mediated dephosphorylation. Future studies will test this hypothesis.

We are grateful to J. Correa, A. Calzada, R. Daga, K. Labib and M. Sacristán for helpful discussions and comments on the manuscript. A.B. received financial support for this research work through PGC and JCyL grants from the Spanish Science and Technology Ministry and Junta de Castilla y León. This research was also supported by The Swiss National Science Foundation, ISREC and PGC of the Spanish Science and Technology Ministry grants to V.S. and S.M. This work was also supported by FPU/MEC and EMBO short-term fellowships to V.E.

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